Amikacin: a Rapid and Sensitive Radioimmunoassay

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A rapid, sensitive, and specific radioimmunoassay has been developed for the measurement of amikacin, a new semisynthetic aminoglycoside antibiotic. Antisera were produced by immunizing rabbits with amikacin conjugated to porcine thyroglobulin. Sera were screened for amikacin antibodies with ^3H -labeled amikacin. Free and bound radioactivity were separated by the second antibody method. As little as 5 ng of the antibiotic can be measured in serum, cerebrospinal fluid, and urine. The standard curve is unaffected by pH (5-11), ionic strength (0.01 M to 0.3 M), MgCl_2 or CaCl_2 (1 to 8 mM/liter), or urea (10-300 mg/100 ml). The antibodies raised to amikacin are not able to distinguish between kanamycin and amikacin. The cross reactivity of all other antibiotics tested was <0.1%. Co-incubation of first and second antibody allows for a 30-min incubation period, making the assay useful for *stat* determinations. The standard curve is linear on a logit-log plot, allowing for rapid computer analysis of the standard curve, interpolation of sample values, and quality-control monitoring.

Radioimmunoassay is a precise, sensitive, and specific analytical technique which has recently become available for the detection and measurement of drugs in biological fluids (5, 6, 10-13).

Amikacin (BB-K8) is a new semisynthetic aminoglycoside antibiotic with a broad spectrum of *in vitro* and *in vivo* antibacterial activity (4, 7). Like other aminoglycosides, amikacin at high doses is potentially ototoxic and nephrotoxic (2). To achieve and maintain therapeutic levels, particularly in individuals with impaired renal function, frequent and rapid monitoring of blood levels is paramount to make valid decisions concerning the dose and dosing intervals.

The present paper reports the development of a rapid radioimmunoassay for amikacin. The assay can be performed in 2 h, requires 5 μliters of serum, is highly specific, and can measure as little as 5 ng of antibiotic.

MATERIALS AND METHODS

Antigen preparation. Amikacin, as a hapten, was conjugated by ethyl carbodiimide (3) to the carrier protein porcine thyroglobulin (Sigma Chemical Co., St. Louis, Mo.). The mole ratio employed in the reaction mixture was 1:500:11,500 of carrier protein to amikacin (potency 864 $\mu\text{g}/\text{mg}$, obtained from Bristol Laboratories, Syracuse, N.Y.) to ethyl carbodiimide (1-ethyl-3[3-dimethylaminopropyl]-carbodiimide hydrochloride, Sigma Chemical Co.), respectively. The

total volume of the reaction mixture was 20 ml. A 100-mg amount of porcine thyroglobulin was dissolved in 5 ml of water and mixed with 5 ml of dissolved amikacin (44 mg) to which had been added 10 μliters (28 μCi) of ^3H -labeled amikacin (tritiated by Amersham-Searle, Arlington Heights, Ill., 14 mCi/50 mg) as a marker of incorporation. A 334-mg amount of ethyl carbodiimide, previously dissolved in 10 ml of water, was added dropwise with constant stirring over a 30-min period at room temperature. The reaction mixture was left at room temperature for 1 h, followed by 24 to 72 h at 4 C.

A 100- μliter sample of the reaction mixture was chromatographed on a Sephadex G-25 column (1 by 10 cm) eluted with phosphate buffered saline (PBS, 0.01 M phosphate-buffered 0.15 M saline, pH 7.5). The ratio of counts in the protein peak to total counts permitted calculation of the mole ratio of incorporation of protein to amikacin. The mole incorporation ratio was 1:115.

The conjugate was dialyzed against 4 liters of PBS daily for 4 days at 4 C.

Immunization. Rabbits were immunized by the Vaitukaitis method (14). Fur was shaved from the back and proximal limbs of five young, adult, female New Zealand albino rabbits. Each rabbit received a total of 100 μg of the synthetic immunogen, emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The emulsion was administered intradermally at 20 sites. At a separate site, 0.5 ml of *Bordetella pertussis* vaccine (Eli Lilly Co., Indianapolis, Ind.) was injected subcutaneously. Beginning with the fifth week after immunization, rabbits were bled by cardiac puncture. Booster injections were given in the same manner as

the initial immunization omitting the *Bordetella* vaccine. Rabbits were boosted every 30 days and bled 10 days thereafter. Serum was separated, aliquoted, and stored at -16°C .

Antisera screening. Sera were screened in serological test tubes (10 by 75 mm), Scientific Products, Santa Ana, Calif.) for the presence of amikacin-binding antibodies with a reaction mixture containing 100 μliters of serum diluted 1:10 with PBS, 100 μliters of 0.1 M ethylenediaminetetraacetic disodium salt to which had been added 1% normal rabbit serum (NRS), 750 μliters of PBS containing 1% NRS and 50 μliters of ^3H -labeled amikacin containing approximately 10,000 counts/min of ^3H . The tubes were vortexed and incubated at 4°C for 24 h. To separate free from bound radioactivity, 100 μliters of sheep anti-rabbit gamma globulin (previously titrated to determine the volume needed for maximum precipitation of first antibody) was added to all tubes and vortexed. The tubes were incubated at 4°C for an additional 24 h and then centrifuged. The bound radioactivity was counted in the precipitate after aspiration. An antiserum which bound 32% of the radioactivity at a final dilution of 1:100 was used in the assay.

Radioimmunoassay. The assay was run in serological test tubes (10 by 75 mm) with 50- μliter , 100- μliter Eppendorf, and 1-ml Oxford automatic pipettors (Scientific Products, Santa Ana, Calif.). For an 11-point standard curve, with high, middle, and low controls, and unknown samples, all in triplicate, the protocol used was as follows.

Five tubes containing 50 μliters of ^3H -labeled amikacin (10,000 counts per min per 50 μliters) were used for total counts. Ten tubes with 50 μliters of ^3H -labeled amikacin, 650 μliters of 1% NRS in PBS, 100 μliters of 1% NRS in ethylenediaminetetraacetic disodium salt, 100 μliters of normal human serum diluted 1:20 with PBS, and 100 μliters of amikacin antisera (1:10 dilution with PBS) were used for zero tubes (maximal binding tubes).

A stock solution of amikacin was prepared by dissolving 10 mg in 100 ml of 1% NRS in PBS. Three working standard solutions were prepared by diluting the stock solution 1:10, 1:100, and 1:1,000 in 1% NRS in PBS. Amounts from 20 to 100 μliters of the appropriate working solution were used to give standard curve points of 1, 2, 4, 6, 8, 10, 25, 50, 100, 250, and 1000 ng (Fig. 1). Standard curve tubes also contained 100 μliters of normal human serum (1:20), 100 μliters of antiserum (1:10) and varying amounts of 1% NRS in PBS to bring the final volume to 1 ml.

Patient sera were diluted 1:20 with 1% NRS in PBS and run in triplicate. A 100- μliter amount of the diluted patient serum was added to 50 μliters of ^3H -labeled amikacin, 100 μliters of 1% NRS in ethylenediaminetetraacetic disodium salt, and 750 μliters of 1% NRS in PBS. A 100- μliter amount of previously titrated sheep anti-rabbit gamma globulin was added to all tubes to precipitate the first antigen-antibody complex. Tubes were vortexed and incubated at 40°C for 10 min, followed by 20 min in a 4°C ice bath. All test tubes except those containing the total counts were centrifuged for 5 min at 2000 to 3000

FIG. 1. Standard curve for the amikacin radioimmunoassay. The ratio of bound to free amikacin is plotted as a percentage of counts precipitated by the second antibody.

$\times g$. The supernatant fluid was aspirated, the precipitate was solubilized with 100 to 250 μliter of Protosol, a tissue and gel solubilizer (New England Nuclear, Boston, Mass.), and the tubes were vortexed until the solution became clear. A 1-ml amount of toluene-Omnifluor (4 g of Omnifluor, New England Nuclear, Boston, Mass., per liter of liquid-scintillation grade toluene) was added, and the solution was poured into vials containing 8 ml of the scintillation cocktail. A 1-ml rinse of the test tube with scintillation cocktail completed the transfer phase. The contents of those tubes containing the total counts were washed and rinsed directly into scintillation vials in the above manner.

Radioactivity was counted in a Beckman LS-333 beta liquid scintillation counter with an efficiency of 43% for tritium. The counter was set to record time to 10,000 counts (2% error).

RESULTS AND DISCUSSION

All five rabbits immunized with the amikacin conjugate produced antibodies at first bleeding. The binding of labeled amikacin ranged from 21 to 56% at a 1:10 initial of the rabbit serum or a 1:100 final solution in the assay system with the labeled amikacin as received without further purification. The standard curve obtained with one of the antisera is shown in Fig. 1.

Two standard deviations below the mean of the counts precipitated in the absence of unlabeled amikacin was used to determine assay susceptibility. Assay susceptibility was 5 ng/tube.

When serum, urine, and cerebrospinal fluid were enriched with increasing amounts of a stock solution of amikacin in PBS, the displacement curves paralleled the standard curve and the recovery curves were linear with a slope of 1. The binding of labeled amikacin was unaffected by variations in pH (5 to 11), ionic strength

(0.01 M to 0.3 M), $MgCl_2$ or $CaCl_2$ (1 to 8 mM/liter), urea (10 to 300 mg/100 ml) or by up to 40% hemolysis of red blood cells. There was a 50% reduction observed in the percentage of counts bound when more than 100 μ liters of bile was added to the assay system.

Maximum binding of labeled amikacin was reached with a first antibody incubation of 10 min at 4 C. Binding was unaffected when first antibody and second antibody were incubated simultaneously. Co-incubation of first antibody with second antibody for 10 min at 40 C followed by 20 min at 4 C achieved maximum binding of labeled amikacin. The total incubation time of 30 min enhances the utilization of this radioimmunoassay for *stat* determinations of amikacin (1).

The rapidity of microbiological assays may be nullified by the presence of certain antibiotics. To test the specificity of the antibody for amikacin, cross-reactivity studies were conducted. The results were expressed as the relative potency of other drugs compared to amikacin, for the 50% displacement of labeled amikacin. The percentage of cross-reactivity was less than 0.1% for all antibiotics tested except kanamycin (Table 1). The antibodies raised to amikacin were not able to distinguish the difference between kanamycin and amikacin which is derived from the acylation of the deoxystreptamine moiety of kanamycin A (4). Further specificity studies showed cross-reactivities of less than 0.1% with a wide variety of other drugs listed as follows: chlorthiazide, metaraminol, cyclophosphoramide, mephentermine, hydrocortisone, digoxin, imuran, chlordiazepoxide, sodium iodide, aminophyllin, pentazocine lactate, levanterenol bitartrate, sodium pheno-

TABLE 1. Relative cross-reactivities of other antibiotics with amikacin-binding antibodies

Compound	Cross-reactivity (%) ^a
Other aminoglycosides	
Neomycin	<0.1
Streptomycin	<0.1
Tobramycin	<0.1
Gentamicin	<0.1
Kanamycin	100.0
Other antibiotics	
Carbenicillin	<0.1
Methicillin	<0.1
Oxytetracycline	<0.1
Cephalothin	<0.1
Chloramphenicol	<0.1

^a As determined by the relative amounts needed for 50% displacement of radioactive amikacin.

barbitol, isoproterenol, chlorpromazine, caffeine sodium benzoate, morphine sulfate, codeine phosphate, merperidine hydrochloride, and mercaptopurine.

With the method of Rodbard et al. (8, 9), the sigmoid-shaped standard curve is linear on a logit-log plot allowing computer analysis of the standard curve and computer interpolation of the sample values. The raw data from the beta counter are simultaneously printed and punched on paper tape by a teletype (model 8470) connected directly to the counter. From the punched paper tape, an EMR 6130 computer and a Calcomp 565 plotter analyze and plot the standard curve giving the 95% confidence limits, interpolate patient values, and render quality control data. Linearizing the standard curve allows for further quality control where accumulation of slope and intercept values can be monitored.

Assay precision was measured on ten replicates of an amikacin-containing serum run in the same assay. The coefficient of variation was 2.9% in a mean value of 23 μ g/ml and the range in values was from 22.0 to 24.0 μ g/ml.

The validity of the radioimmunoassay for amikacin was further established by comparison with results obtained by microbiological techniques and a radioenzymatic method. Nineteen patient samples were measured by all three methods (Table 2). The correlation coefficient

TABLE 2. Amikacin determinations^a

Sample	Microbiological assay	Radioenzyme assay	Radioimmunoassay (RIA)
S-1	16.8	17	15
S-2	29.4	28.5	28
S-3	24.3	24	32
S-4	16.8	18	18
AA97	22.5	21.5	15
AA98	11.7	15	15
AB1	31.8	36	34
AB2	12.6	14.5	11
AB4	26.4	28	32
AB5	39	33.5	33
AB7	21	17.5	21
AB9	36	33.5	40
AB10	1.7	1.5	1.3
AB11	19.5	16	15.2
AB12	10.8	12	14
AB14	29.1	35	30
AB15	39	44	43
AB16	25.2	29.5	32
AB17	1.35	2.3	1.5

^a Correlation coefficients of assays: radioenzyme vs. RIA = 0.96; microbiological vs. RIA = 0.93; and microbiological vs. radioenzyme = 0.96.

cient between microbiological and radioimmunoassay values was 0.93. Comparison of the same 19 samples by radioenzymatic and radioimmunoassay gave a correlation coefficient of 0.96.

The excellent correlation observed with all three methods should encourage clinicians to utilize an assay method most applicable to their laboratory facilities. Frequent and accurate measurements of this potentially toxic antibiotic permits its use with greater effectiveness and safety. Because the radioimmunoassay of amikacin is partially automated and computerized, it can be adapted to large numbers of samples. It provides a practical, sensitive and rapid method for monitoring this new antibiotic.

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